Remarks

Claims 63-78 are pending in the subject application. By this Amendment, Applicants have amended claims 63, 66-70 and 73-76. Support for the amendments claims can be found throughout the subject specification and in the claims as originally filed (see, for example, page 43, line 10 through page 46, line 24). Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 63-78 are currently before the Examiner. Favorable consideration of the pending claims is respectfully requested.

Applicants note that the Office Action indicates that the oath or declaration in this matter is defective because the foreign priority must be disclosed in the oath/declaration. Applicants respectfully submit that the foreign priority information for this application (under 35 U.S.C. § 119) was identified on the Application Data Sheet (ADS) when the subject application was filed and on the Supplemental ADS filed August 31, 2005. Applicants respectfully submit that the filing of the ADS fulfills the requirements of identifying the foreign priority under 37 C.F.R. § 1.76(b)(6). Accordingly, it is respectfully submitted that the requirements for properly claiming foreign priority in this matter have been met and acknowledgment of the same is respectfully requested.

The Examiner has indicated that the title of the invention is not descriptive and that a new title is required that is clearly indicative of the invention to which the claims are drawn. Applicants have amended the title of the invention to "A Polypeptide Having Kinase Activity or Which Activates the MAP Kinase Pathway." Accordingly, reconsideration and withdrawal of this objection is respectfully requested.

The abstract of the disclosure of the subject specification has been objected to for not completely describing the disclosed subject matter. Applicants have amended the abstract in accordance with the Examiner's suggestion. Applicants respectfully submit that no new matter has been incorporated in this Abstract. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

The application is also objected to on the grounds that the subject specification fails to comply with the requirements of 37 CFR 1.821(a)(1) and (a)(2). Specifically, the Examiner indicates that Figures 2, 4, and 6-9 contain polypeptide or nucleic acid sequences without appropriate SEQ ID NOs. A replacement sequence listing is attached that provides sequence identifiers for polypeptides

identified as the top blastp alignment result from the non-redundant protein database. Applicants further note that section 2422.02 of the M.P.E.P. indicates that:

> In view of the fact that many significant sequence characteristics may only be demonstrated by a figure, the exclusive conformance requirement of this section may be relaxed for drawing figures. This is especially true in view of the fact that the representation of double stranded nucleotides is not permitted in the "Sequence Listing" and many significant nucleotide features, such as "sticky ends" and the like, will only be shown effectively by reference to a drawing figure. Further, the similarity or homology between/among sequences can only be depicted in an effective manner in a drawing figure.

In light of this guidance, Applicants note that the alignment presented in the line between the Query and Subject lines in Figures 2, 4, and 6 represents the depiction of the alignment result and that the sequence rules do not provide for an adequate means of presenting such a sequence in a sequence listing. As stated in section 2423.03 of the M.P.E.P.:

> In 37 CFR 1.822(e) the procedures for presenting and numbering hybrid and gapped sequences are set forth. A sequence that is made up of one or more non-contiguous segments of a larger sequence or segments from different sequences, *i.e.*, a hybrid sequence, shall be presented as a separate sequence. A "gap" for the purpose of this section is not intended to embrace a gap or gaps that is/are introduced into the presentation of otherwise continuous sequence information in, *e.g.*, a drawing figure, to show alignments or similarities with other sequences. The "gaps" referred to in this section are gaps representing unknown or undisclosed regions in a sequence between regions that are known or disclosed.

Thus, Applicants have no means by which the gaps in the sequence depicting the alignment sequence can be presented and respectfully request that the Examiner relax absolute conformance with the Sequence Rules (only with respect to the sequences depicting the alignment sequences presented within the figures). Applicants respectfully assert that no new matter has been added by any of the amendments and submit that the Brief Description of the Figures section has been amended to identify the sequence identifiers in Figures 2, 4, and 6-9. Accordingly, reconsideration and withdrawal of the objection is respectfully requested.

Claims 63-78 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite in the use of the limitation "the activity of a germinal center kinase." Applicants respectfully assert that the claims as filed are definite. However, by this Amendment, the claims have been amended to indicate

that the polypeptide has "kinase activity or activates the MAP kinase pathway". Support for the amendment can be found, for example, at page 45, lines 19-21 of the as-filed specification. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, second paragraph, is respectfully requested.

Claims 63, 66-70, and 73-76 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Applicants respectfully assert that there is adequate written description in the subject specification to convey to the ordinarily skilled artisan that they had possession of the claimed invention. Specifically, use of the recitation "over its full length to the amino acid sequence ..." is rejected on the basis that language allows for an interpretation that includes an interpretation wherein the specified full length can be as few as two consecutive amino acids. By this Amendment, the claims have been amended to delete reference to "over its full length to the amino acid sequence" and to indicate that the claimed isolated polypeptide has at least 95% identity to SEQ ID NO: 100 and has kinase activity or activates the MAP kinase pathway. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 63, 66-70, and 73-78 are rejected under 35 U.S.C. § 112, first paragraph, as nonenabled by the subject specification. The Office Action indicates that the specification is enabled for a method for producing a polypeptide of SEQ ID NO: 100, but is not enabled for any polypeptide with any germinal center kinase nor is the specification enabling for polypeptide fragments that is a portion or fragment of SEQ ID NO: 100 and that has that activity of a germinal center kinase. Applicants respectfully assert that the claims are enabled by the subject specification. However, as indicated above, the phrase "activity of a germinal center kinase" has been deleted from the claims and the claims now recite a polypeptide that has kinase activity or activates the MAP kinase pathway and the language related to the percent identity for the claimed polypeptide relates to the amino acid sequence of SEQ ID NO: 100. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claims 63-78 are rejected under 35 U.S.C. § 101 on the grounds that the claimed invention lacks patentable utility. The Office Action argues that instant specification discloses, at page 21:

> In a tenth aspect, the invention provides for the use of a polypeptide of the first aspect of the invention as a Germinal Center Kinase (GCK), preferably as a NIK-like kinase and more preferably as a NIK-like embryo specific kinase (NESK). Suitable uses of the polypeptides of the invention as Germinal Center Kinases (GCK), preferably as NIK-like kinases and more preferably as NIK-like embryo specific kinases (NESK) include use as a regulator of cellular growth, metabolism or differentiation, use as part of a receptor/ligand pair and use as a diagnostic marker for a physiological or pathological condition.

The Office Action continues that the instant specification does not disclose any significant utility for the claimed polypeptides. Applicants traverse.

Applicants' respectfully submit that the as-filed specification provides a specific, substantial, and credible utility as well as a well-established utility for the claimed invention, in addition to those described in the passage referred to in the Office Action. As noted in the as-filed specification, the claimed polypeptides can also be used to identify agonists or antagonists of the polypeptide's activity (*e.g.*, kinase activity or the ability to activate the MAP kinase pathway; see, for example, page 43, line 10 through page 46, line 24). As was known at the time the subject application was filed (circa September 2002), the MAP kinase pathway was a therapeutic target for the development of therapeutic agents for the treatment of disease (see, for example, Sebolt-Leopold, *Oncogene*, 2000, 19:6954-6599, "Development of anticancer drugs targeting the MAP kinase pathway", a copy of which is attached for the Examiner's convenience). As such, the claimed polypeptides are not only useful as asserted above, but the polypeptides are also useful in screening assays for the identification of compounds capable of targeting the MAP kinase pathway that can be used in the development of anticancer agents. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 101 is respectfully requested.

Claims 63-78 are rejected under 35 U.S.C. § 102(b) as anticipated over Nakano *et al.* (2000). The Office Action indicates that the Nakano *et al.* reference teaches a polypeptide NESK, a member of germinal kinase, comprising an amino acid sequence wherein said sequence of its full length is 100% identical to a corresponding sequence of SEQ ID NO: 100. Applicants respectfully assert that the Nakano *et al.* reference does not anticipate the claimed invention as it fails to teach a polypeptide

that has at least 95% identity to SEQ ID NO: 100. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 102(b) is respectfully requested.

It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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Sebolt-Leopold, *Oncogene*, 2000

Oncogene (2000) 19, 6594–6599

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Development of anticancer drugs targeting the MAP kinase pathway

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Since the discovery of the role of ras oncogenes in tumorigenesis, we have witnessed an explosion of research in the signal transduction area. In the quest to understand how Ras transmits extracellular growth signals, the MAP kinase (MAPK) pathway has emerged as the crucial route between membrane-bound Ras and the nucleus. The MAPK pathway encompasses a cascade of phosphorylation events involving three key kinases, namely Raf, MEK (MAP kinase kinase) and ERK (MAP kinase). This kinase cascade presents novel opportunities for the development of new cancer therapies designed to be less toxic than conventional chemotherapeutic drugs. Furthermore, as a signal transduction-based approach to cancer treatment, inhibition of any one of these targets has the potential for translational pharmacodynamic evaluation of target suppression. The rationale for targeting the MAP kinase pathway will be reviewed here along with a discussion of various pharmacological approaches and the promise they hold for a new generation of anticancer drugs. *Oncogene* (2000) **19,** 6594–6599.

Keywords: mitogen-activated protein kinase (MAPK); extracellular signal-regulated kinase (ERK); MAP kinase kinase (MEK); raf

Introduction

Many receptor tyrosine kinases and cytokine receptors in association with heterotrimeric G proteins are known to activate intracellular protein serine/threonine kinases termed mitogen-activated protein kinases (MAPKs). Of the various families of MAPKs, which are also referred to as extracellular signal-regulated kinases (ERKs), the first to be characterized were ERK1 and ERK2. Both of these ERKs are activated in response to diverse extracellular stimuli and by protooncogene-encoded proteins that induce proliferation. A cascade of phosphorylation events downstream from Ras activates these kinases. Upstream regulation of the MAP kinase pathway is complex as evidenced by the number of functions fulfilled by its activation. Processes impacted by MAPK activation encompass the cytoplasm, nucleus, cytoskeleton, and the membrane. The reader is referred elsewhere for comprehensive reviews on the subject of regulation through MAPK cascades (Cobb, 1999; Lewis *et al*, 1998; Kolch, 2000).

The Raf-MEK-ERK pathway represents one of the best characterized Ras signaling pathways. Raf and MEK have consequently emerged as key protein kinases to target for anticancer drug design. While there exist multiple MAP kinase families, e.g. jun kinase and p38, which are also activated downstream of low molecular weight G-proteins, ERK has been the best characterized and is more pertinent to aberrant signaling in human cancer. For some cancers, especially those of hematopoietic origin, the p38 and jun kinase pathways may in fact yield targets exploitable for anticancer drug development. However, a broad array of solid tumors is known to express constitutive levels of phosphorylated ERK1 and ERK2. Activation of ERK is critical for a large number of Ras-induced cellular responses. Included among these responses is transcriptional activation of multiple genes (Hill and Treisman, 1995). The best-characterized physiological substrates of ERK are ternary complex factors (TCFs), which are directly phosphorylated by ERK to activate their transcription activation potential (Gille *et al*., 1992; Janknect *et al*., 1993; Marais *et al*., 1993). TCFs, in association with serum response factor, is thought to be critical for the activation of numerous mitogen-inducible genes (Hill and Treisman, 1995).

Many molecules ultimately contribute to activation of the Ras-ERK pathway, including a number that are involved in protein-protein interactions. With respect to pharmacological intervention, it is generally difficult to selectively target the binding site shared by two proteins. It is therefore not coincidental that the development of agents targeting the Ras-MAPK pathway has largely focused on the design of small molecule inhibitors of enzyme function. As will be explored in more detail below, four proteins have emerged as key players in the quest to intervene in this pathway: Ras, Raf, MEK (MAP kinase kinase), and ERK. Ras is the subject of a paper that appears elsewhere in this review issue and therefore will not be covered further here.

Rationale for targeting the MAP kinase pathway

Figure 1 provides a simplified schematic representation of the signaling events leading to activation of the MAP kinase pathway. Initially, Ras interacts with and activates the serine/threonine protein kinase Raf1 in a GTP-dependent manner (Daum *et al*., 1994; Stokoe *et al*., 1994). A family of Raf protein kinases has been identified and is comprised of A-Raf, B-Raf, and c-Raf1. It has been suggested that this family of kinases, which is known to regulate proliferation, differentiation, and apoptosis, have both overlapping and unique regulatory functions (Hagemann and Rapp, 1999). For example, transfection of oncogenic H-ras led to a preferential activation of endogenous c-Raf1 as opposed to A-Raf (Weber *et al*., 2000). Mutated Raf-1 is constitutively active and possesses *in vitro* transforming potential (Stanton and Cooper, 1987). The potential for Raf-1 to play a broad role in tumorigenesis is evidenced by its ability to become activated by either PKCα or the antiapoptotic protein Bcl-2 in a Ras-independent manner (Kolch *et al*., 1993; Wang *et al*., 1996).

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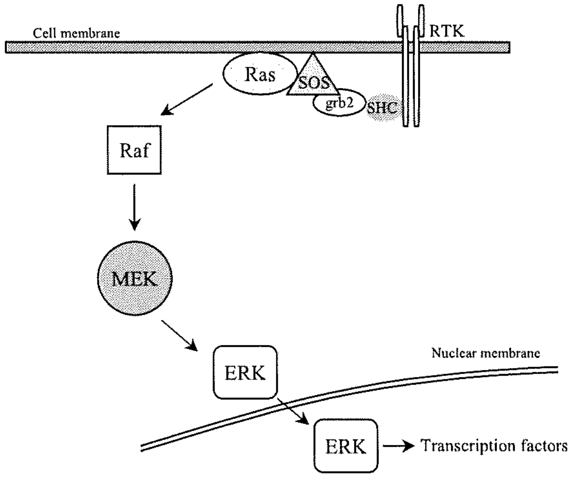

Figure 1 Schematic representation of the Ras–MAP kinase pathway. The MAP kinase cascade contains three sequential kinases: a MAP kinase kinase kinase (Raf), MAP kinase kinase (MEK), and MAP kinase (ERK)

Importantly, *raf* mutations have been identified in a range of human tumors (Storm and Rapp, 1993). Independent of its mutation status, Raf is also activated in tumor cells containing enhanced growth factor signaling pathways, such as those induced by mutant or constitutively expressed Ras or EGF receptor family members. Therefore, the collective evidence suggests that Raf-1 is a viable anticancer drug target.

Alternatively, targeting the molecule immediately downstream from Raf, that is, the dual specificity kinase MEK can also be envisioned as representing a rational approach to anticancer drug design. Subsequent to its activation, Raf-1 phosphorylates and activates both MEK1 and MEK2 (hereafter referred to as MEK) on two distinct serine residues (Dent *et al.*, 1992; Crews *et al.*, 1992; Her *et al.*, 1993). Activated MEK then phosphorylates ERK1 and ERK2 on both a tyrosine and a threonine residue (Anderson *et al.*, 1990). No substrates for MEK have been identified other than ERK1 and ERK2 (Seger *et al.*, 1992). This tight selectivity in addition to the unique ability to phosphorylate both tyrosine and threonine residues are consistent with this kinase playing a central role in integration of signals into the MAPK pathway. Constitutive activation of MEK has been shown to result in cellular transformation (Cowley *et al.*, 1994; Mansour *et al.*, 1994). While MEK has not been identified as an oncogene product, MEK is the focal point of many signal transduction mitogenic pathways activated by proven oncogenes. Pivotal studies carried out with the MEK inhibitor PD98059 provided further impetus for exploring whether MEK could be exploited as a target for rational anticancer drug design. In these studies, MEK inhibition not only impaired proliferation, but also impacted a diverse array of cellular events, including differentiation, apoptosis, and angiogenesis (Dudley *et al.*, 1995; Alessi *et al.*, 1995; Pages *et al.*, 1993; Pang *et al.*, 1995; Finlay *et al.*, 2000; Holmstrom *et al.*, 1999; Elliceiri *et al.*, 1998; Milanini *et al.*, 1998). Based on these collective findings, MEK therefore represents an attractive target for pharmacological intervention in cancer.

Theoretically, it could be argued that intervention in any of the kinase events in the MAPK cascade could represent a viable approach to crippling tumor growth. If so, then Raf-1, MEK, and ERK all emerge as reasonable anticancer drug targets. The advent of high volume screening of pharmaceutical libraries for small molecule inhibitors has most certainly produced reasonable drug candidates targeting all steps of this pathway. For example, a cascade assay has been reported that is capable of identifying inhibitors of cRaf1, MEK1, or ERK2 (McDonald *et al*, 1999). As we now turn to preclinical and clinical evaluation of these small molecule inhibitors, it is important to keep in mind that their ultimate promise or differences may depend as much on their pharmacological attributes as on the merits of their targeted kinase.

It should be noted that the identification of pathway components in the Ras-MAP kinase pathway is likely incomplete. For example, a Raf-1-interacting protein, RKIP, has recently been reported (Yeung *et al.*, 1999). This protein inhibits the phosphorylation and activation of MEK by Raf-1 and has also been shown to co-localize with Raf-1. It has been proposed that RKIP binding to either Raf-1 or MEK dissociates Raf-MEK complexes, thereby interrupting MEK activation and downstream signaling (Yeung *et al.*, 2000). Discovered with the use of a yeast two-hybrid system, the relevance of RKIP expression to signal transduction in tumor cells is unclear at the present time. Furthermore, until we learn whether RKIP expression is negatively regulated, it remains unclear how to pharmacologically elevate its expression to impair tumor growth. Although highly speculative based on our current knowledge of the role of RKIP, it is conceivable that elevated expression of this protein could offer tumor cells a mechanism of resistance to MAPK pathway inhibitors.

There exist a multitude of other newly discovered proteins that may provide insight into the design of novel signal transduction-based cancer therapies that exploit the MAP kinase pathway. These include Sur-8, which is thought to act as a scaffold to enhance Ras-MAP kinase signaling by facilitating Ras-Raf interaction (Li *et al.*, 2000), as well as the kinase suppressor of Ras (KSR). KSR is also thought to act as a scaffolding protein for the Ras-MAPK pathway (Stewart *et al.*, 1999). Another interesting protein is MP-1, which has been reported to enhance activation of the MAPK by binding MEK (Schaeffer *et al.*, 1998). Last but not least, a novel ERK has recently been identified, ERK1b, which is an alternatively spliced form of ERK1, that appears to be elevated in Ras-transformed cells (Yung *et al.*, 2000).

Amenability of the MAP kinase pathway to pharmacodynamic evaluation

Using an antibody specific for dually phosphorylated ERK1 and ERK2, *in vivo* evaluation of MEK inhibition can easily be measured in excised samples. The utility of such an assay in preclinical animal models was demonstrated for the MEK inhibitor PD184352 (Sebolt-Leopold *et al.*, 1999). Phosphorylated MAPK is the product of MEK activity and thus represents a direct measure of MEK inhibition. Using an antibody specific for phosphorylated MEK, *ex vivo* evaluation for Raf inhibition should likewise be straightforward. However, pharmacodynamic evalua-

tion of ERK inhibitors would be complex, as multiple nuclear proteins and transcription factors are substrates for phosphorylated ERK.

At the preclinical stage, pharmacodynamic assays are not only useful for optimizing the design of dosing regimens, but also offer the advantage of being able to correlate antitumor efficacy with inhibition of the biochemical target. A large number of cell lines as well as primary human tumors have been surveyed for constitutive activation of the MAPK pathway (Hoshino *et al.*, 1999). It will be of interest to correlate the degree of target expression of a given tumor with its inherent sensitivity to agents directed against that target. The data obtained thus far with the MEK inhibitor PD184352 suggest that tumors containing high level expression of phosphorylated MAP kinase are most sensitive to treatment with this agent (Sebolt-Leopold *et al.*, 1999). There are obvious clinical implications if this pattern continues during the expansion of our database; such assays could then be exploited as prognostic tools to identify those patients most likely to derive therapeutic benefit from treatment with a given agent.

Figure 2 demonstrates the applicability of pharmacodyamic evaluation of PD184352 to a range of tissues or cells (Sebolt-Leopold, unpublished data). Twenty-four hours after an oral dose of 200 mg/kg was administered to monkeys, significant inhibition of MAPK phosphorylation was observed in lung as well as skin tissue (Figure 2a,b, respectively). Looking ahead to the clinical setting, biomarker evaluation of phosphorylated MAPK levels can also be measured in PMA-stimulated peripheral blood mononuclear cells (Figure 2c). Such assays have the potential to define a dose threshold that delivers total suppression of the desired target. For a target such as MEK that is thought to offer tumor-specific pharmacologic effects, Phase II trials may not need to be carried out at the MTD determined from Phase I studies.

Pharmacological approaches to targeting the MAPK pathway

The only Raf-directed approach for which preclinical efficacy data have been published is that employing a c-raf-1 antisense oligonucleotide. ISIS 5132 is a 20-base phosphorothioate antisense oligodeoxynucleotide designed to hybridize to 3′ untranslated sequences of c-raf-1 mRNA (Monia *et al.*, 1996). Reduction of c-raf-1 mRNA was shown to occur in tumor-bearing mice treated with relatively low doses. Importantly, preclinical efficacy and toxicology studies suggested a large therapeutic window for ISIS 5132 (Henry *et al.*, 1997). Early clinical data have recently been reported with ISIS 5132 (Stevenson *et al.*, 1999; Yuen and Sikic, 2000). This agent was well tolerated and suppression of target gene expression was observed in peripheral blood mononuclear cells (O'Dwyer *et al*, 1999). However, Phase II data have not yet been published.

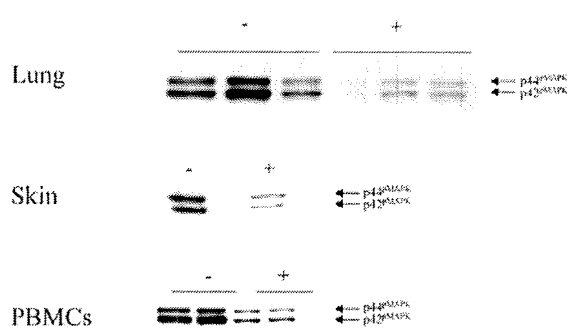

Figure 2 Effects of the MEK inhibitor PD184352 on phosphorylated MAP kinase (pMAPK) levels in (**a**) monkey lung, (**b**) monkey skin, and (**c**) human peripheral blood mononuclear cells (PBMCs). Monkeys were administered an oral dose of 200 mg/mg PD184352 (+) or diluent (−) followed 24 h later by excision of the indicated tissue for analysis of pMAPK. Human whole blood was spiked with 1 μM PD184352 followed immediately by stimulation with PMA and isolation of PBMCs for evaluation of pMAPK levels

It is anticipated that clinical data will soon emerge from testing of small molecule inhibitors of raf kinase. Based on the patent literature, several classes of substituted ureas have been identified as raf kinase inhibitors (Bayer, 1999a,b,c, 2000). Benzamides have also been investigated for their raf kinase inhibitory activity (Zeneca, 1998). It has been reported that a potent and specific inhibitor of Raf isoforms *in vitro*, ZM 336372 paradoxically induces significant activation of c-Raf without inducing any activation of MEK1 or ERK2 (Hall-Jackson *et al.*, 1999). The authors speculate that Raf may suppress its own activation by virtue of a novel feedback loop. If so, then inhibition would be counterbalanced by reactivation which would limit the utility of raf kinase inhibitors as anticancer agents. Clinical testing of raf kinase inhibitors will likely clarify this paradox. It should also be noted that growth factor-stimulated ERK is capable of retrophosphorylating MEK in a negative feedback fashion (Brunet *et al.*, 1994). Yet, MEK inhibitors, e.g. PD184352, clearly exhibit promising preclinical activity in a number of human and murine tumor models. This suggests that the retrophosphorylation-derived negative regulation does not inactivate the pathway.

An orally active small molecule inhibitor of MEK has provided *in vivo* validation for targeting MEK for anticancer drug design (Sebolt-Leopold *et al.*, 1999). In this study, PD184352, a non-ATP-competitive, highly selective inhibitor of MEK, was found to significantly inhibit growth of colon carcinomas of both mouse and human origin. Importantly, efficacy was achieved at well tolerated doses and was correlated with a reduction in the levels of activated MAPK in excised tumors. In addition to impairing tumor proliferation, PD184352 was found to block the disruption of cell-cell contact and motility required for invasion. This finding is consistent with earlier reports indicating that hepatocyte growth factor (HGF) induces dispersion of epithelial cells by a Ras-dependent mechanism. The MEK/MAPK pathway is an essential mediator of HGF-induced cell scattering (Ridley *et al.*, 1995; Herrera, 1998; Potempa and Ridley, 1998; Tanimura *et al.*, 1998). PD184352 (now designated CI-1040) is presently undergoing Phase I evaluation in cancer patients.

Once activated, a fraction of cytoplasmic ERK1 and ERK2 translocates into nuclei (Lenormand *et al*, 1993). In this way, these MAP kinases enable the regulation of gene expression by phosphorylation of nuclear transcription factors. While selective ERK1/ERK2 inhibitors have not been described in the literature,

intervention in activation of transcription factors may prove to be an exploitable approach for anticancer drug development. Until ERK inhibitors are evaluated, the pharmaceutical attractiveness of this kinase is left to speculation. It is not clear whether direct inhibition of ERK would prove to be more toxic than inhibition of the upstream kinases Raf and MEK. Whereas a null mutation in the MEK1 gene proved to be embryonic lethal, ERK1 knockout mice were viable and of normal size (Giroux *et al.*, 1999; Pages *et al* 1999). However, in the case of the p38 MAP kinase family, it has been shown that p38 null mutants result in an embryonic lethal phenotype, unlike the case for MKK3 knockouts (Allen *et al.*, 2000; Lu *et al.*, 1999). Aside from unresolved theoretical concerns regarding potential toxicities, *a priori* ERK and MEK inhibitors might be expected to act similarly since ERK can only be activated by MEK. This is in contrast to the situation in the p38 pathway where p38 can be activated by three distinct MKKs. Therefore, to target the pertinent upstream MAP kinase kinases in the p38 pathway, it might prove necessary to abolish activity of not one but three enzymes, namely MKK3, MKK4, and MKK6.

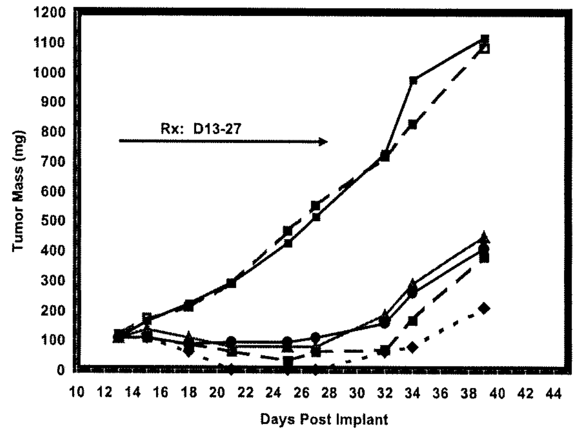

Figure 3 Effects of oral PD184352 (CI-1040) treatment on growth of staged pancreatic BxPc3 xenografts. Treatments of mice bearing subcutaneous implants of BxPc3 tumors was initiated when tumors reached 100 mg in size. PD18452 was administered orally three times a day on days 13 through 26 post-implantation. Doses administered were 200 mg/kg (◆), 124 mg/kg (■, dashed line), 77 mg/kg (●), and 48 mg/kg (▲). Controls plotted here included untreated animals (□) and diluent-treated animals (■, solid line)

Blockade of the MAP kinase pathway exerts pleiotropic effects exploitable in future clinical trial design

Of the numerous therapeutic approaches to cancer treatment, most take the form of a single-pronged attack aimed at either: (1) slowing of tumor growth, (2) inhibition of invasion and metastasis, (3) induction of tumor cell death, or (4) promotion of tumor differentiation. However, by pharmacological intervention of the MAP kinase pathway, one can envision a single agent that concurrently exploits more than one of these processes. Perhaps the best evidence in support of this statement is provided by the collective data obtained with MEK inhibitors.

Greater than 1500 references appear in the literature describing the utility of PD98059 in elucidating the role of the MAP kinase pathway in diverse cellular processes. The involvement of this pathway in tumor proliferation is well documented. While not all tumors rely on MAP kinase activation to drive their growth, a significant percentage of human tumors do in fact exhibit constitutive activation of the MAPK pathway (Hoshino *et al.*, 1999). MEK inhibition has been shown to effectively shut down tumor growth *in vivo* in a cytostatic manner (Sebolt-Leopold *et al.*, 1999). However, MEK inhibition has also proven to induce tumor regressions in some xenograft models, e.g. pancreatic BxPc3, as exemplified in Figure 3 (Merriman and Sebolt-Leopold, unpublished data). These results are consistent with an increase in apoptosis occurring in response to MEK inhibition. This is perhaps not surprising in view of evidence that one of the phosphorylation sites on the pro-apoptotic molecule BAD, i.e. serine-112, is phosphorylated by MAP kinase (Fang *et al.*, 1999; Scheid *et al.*, 1999). Phosphorylation of this site results in loss of the ability of BAD to heterodimerize with the survival protein BCL-2. Thus, by promoting interaction between BAD and BCL-2, it appears feasible that inhibition of MEK would serve to increase the incidence of apoptosis. In BxPc3 tumors that had regressed in response to treatment with the MEK inhibitor CI-1040, reduced phosphorylation of the serine-112 site on BAD was demonstrated *ex vivo* (Sebolt-Leopold, unpublished results). It should also be noted that a recent report indicates that activation of the MAPK pathway acts to protect pancreatic tumor cells from apoptosis by regulating expression of Bcl-2 (Boucher *et al.*, 2000).

Inhibition of MAP kinase signaling is also anticipated to result in anti-metastatic and anti-angiogenic effects. Activation of the MAPK pathway occurs in response to integrin-mediated cellular adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis (Chen *et al.*, 1994; Zhu and Assoian, 1995). It was recently reported that active ERK is targeted to newly formed focal adhesions after integrin engagement of v-Src activation, providing support for a role for ERK in regulation of adhesion (Fincham *et al.*, 2000). Transfection of constitutively active MEK, which resulted in increased expression of matrix metalloproteinases 2 and 9 as well as cathepsin L, resulted in macroscopic metastases (Welch *et al.*, 2000). It is therefore not surprising that MEK inhibition in colon tumor models resulted in decreased invasiveness as well as inhibition of cell motility (Sebolt-Leopold *et al.*, 1999). It is also anticipated that inhibition of MAPK signaling will negatively impact angiogenesis. Such an effect is likely based on our knowledge of sustained activation of MAPK being required for angiogenesis (Eliceiri *et al.*, 1998). MAPK activation is probably also required for growth factor-induced secretion of angiogenic growth factors from tumor cells (Petit *et al.*, 1997).

Therefore, evidence would seem to suggest that single agent treatment with a drug targeted against the MAPK pathway could potentially impair tumor survival by more than one of the therapeutic approaches outlined above. It is likely however that the design of future clinical trials with MAPK pathway

inhibitors will attempt to boost therapeutic kill by employing combination regimens. Two classes of chemotherapeutic agents of particular interest in this regard are mitotic inhibitors, e.g. taxanes, as well as platinum-coordination complexes, e.g. cisplatin and carboplatin. The kinetochore motor protein CENP-E, which was found *in vivo* to associate preferentially with active MAPK during mitosis, was also phosphorylated by MAPK at sites known to regulate its interactions with microtubules (Zecevic *et al.*, 1998). These investigators propose that MAP kinase may play a role in mitosis by affecting the ability of CENP-E to mediate interactions between microtubules and chromosomes. Cell culture experiments have shown that the combination of taxol with the MEK inhibitor CI-1040 results in a significant increase in apoptotic frequency that is greater than that predicted from the additive effects of each agent tested alone (Sebolt-Leopold, unpublished data).

With respect to platinum coordination complexes, cisplatin treatment of ovarian carcinoma cells or HeLa cells has been reported to result in induction of ERK activity (Persons *et al.*, 1999; Wang *et al.*, 2000). Furthermore, inhibition of cisplatin-induced ERK activity by the MEK inhibitor PD98059 resulted in enhanced cytotoxicity in response to cisplatin treatment. Thus the combination of cisplatin or carboplatin with MAPK pathway inhibitors warrants further investigation for potential clinical benefit. The p53 phenotype may play a role in determining whether the combination of a MAPK signaling antagonist with a cytotoxic agent results in synergistic cell kill, since a link has been established between p53 signaling and the MAPK cascade. It has been reported that treatment of normal cells with DNA-damaging agents induced ERK activation in a p53-dependent manner, whereas tumor-derived p53 mutants that were defective in DNA-binding failed to activate ERK (Lee *et al.*, 2000). Interestingly, it was recently reported that inhibition of ERK activation by MEK inhibition resulted in decreased accumulation of p53 during exposure to cisplatin (Persons *et al.*, 2000). These investigators further showed that p53 was phosphorylated by ERK *in vitro* in an event antagonized by MEK inhibition during cisplatin treatment. Thus it appears likely that ERK activation induced by cisplatin regulates the p53 response to cytotoxic damage induced by this DNA-damaging agent.

While only two examples have been given here, one could rationalize the combination of a MAPK pathway inhibitor with a multitude of other agents. If single agent treatment with MAPK signaling antagonists proves to be well tolerated upon chronic dosing, it is tempting to speculate that these drugs might also prove useful in preventing the emergence of hormone-resistant cancers. For example, estrogen-dependent breast cancers that initially respond to tamoxifen treatment frequently become resistant. It has been shown that this shift in hormone-response pattern is accompanied by a shift from MAPK-independent to MAPK-dependent cell growth (Lange *et al.*, 1996). Along these same lines, data exist in support of increased activation of the MAP kinase pathway as prostate cancer progresses to a more advanced and androgen-independent state (Gioeli *et al.*, 1999).

Looking to the future

The ultimate therapeutic promise of signaling antagonists directed against the MAPK pathway can only be determined from human testing. Until we have gained clinical experience on their safety and efficacy profiles, arguing the merits of targeting one kinase versus another remains an academic exercise. Clearly, the chances for clinical success will be enhanced if human trials are designed to exploit the mechanism of action of the agent under study. Combination regimens employing chemotherapeutic agents have generally been driven by safety considerations, i.e. combination of cytotoxic agents with non-overlapping toxicities. With the development of signaling antagonists that are considerably less toxic, it will be important to turn our attention to combining MAPK pathway inhibitors with cytotoxic agents or with other signaling antagonists based on anticipated mechanistic-based synergy.

As these agents enter the clinic, reagents will be available to directly monitor target suppression. The use of such biomarker analysis will not only aid dose escalation, but also offers the advantage of correlating efficacy with the degree of activity anticipated by the extent of target suppression. The advantages of having pharmacodynamic assays available for analysis of clinical samples can not be overstated. The field of clinical oncology is plagued by examples of negative clinical trials, where it is not clear if lack of efficacy was due to the inhibited target being inconsequential to outcome or simply whether the requisite degree of target inhibition was not achieved. Furthermore, if retrospective data analysis from clinical trials shows that the degree of target expression correlates well with sensitivity to the test agent, then this has obvious prognostic implications when tumor biopsy material is available.

In summary, the next decade will no doubt represent a very exciting time in the field of clinical oncology, as a number of signaling antagonists, including inhibitors of the MAP kinase pathway, get put to the real test.

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